

A Novel TNF Receptor Family Member Binds TWEAK and Is Implicated in Angiogenesis

Steven R. Wiley^{1,4}, Linda Cassiano¹, Timothy Lofton¹,
Terry Davis-Smith¹, Jeffrey A. Winkles²,
Volkhard Lindner³, Hua Liu¹,
Thomas O. Daniel¹, Craig A. Smith¹,
and William C. Fanslow¹

¹Immunex Corporation
51 University Street
Seattle, Washington 98101

²Department of Vascular Biology
Holland Laboratory
American Red Cross
Rockville, Maryland 20855

³Center for Molecular Medicine
Maine Medical Center Research Institute
Scarborough, Maine 04106

Summary

TWEAK is a member of the TNF ligand family that induces angiogenesis *in vivo*. We report cloning of a receptor for TWEAK (TweakR) from a human umbilical vein endothelial cell (HUVEC) library. The mature form of TweakR has only one hundred and two amino acids and six cysteine residues in its extracellular region. Five different assays demonstrate TWEAK-TweakR binding, and the interaction affinity constant (K_d) is within a physiologically relevant range of 2.3 ± 0.1 nM. The TweakR cytoplasmic domain binds TRAFs 1, 2, and 3. Cross-linking of TweakR induces HUVEC growth, and mRNA levels are upregulated *in vitro* by a variety of agents and *in vivo* following arterial injury. Soluble TweakR inhibits endothelial cell migration *in vitro* and corneal angiogenesis *in vivo*.

Introduction

TWEAK is a member of the TNF family of cytokines that was first described as a weak inducer of apoptosis in transformed cell lines (Chicheportiche et al., 1997). TWEAK induces IL-8, IL-6, and ICAM-1 expression in cultured human astrocytes (Saas et al., 2000), and cultured monocytes have been shown to respond to IFN- γ by inducing surface expression of TWEAK (Nakayama et al., 2000). TWEAK mRNA expression is also downregulated in murine models of inflammation (Chicheportiche et al., 2000). In addition, TWEAK induces proliferation of human smooth muscle and endothelial cells *in vitro*, and acts as a potent inducer of angiogenesis in a rat cornea pocket angiogenesis assay (Lynch et al., 1999). Given the involvement of TWEAK in inflammation models and its expression pattern on monocytes it is important to identify the receptor for TWEAK in order to better elucidate its role in the immune as well as angiogenic systems.

It was reported previously that TWEAK binds to DR3, a member of the TNF receptor family that contains a

death domain in its cytoplasmic tail and can induce apoptosis (Marsters et al., 1998). We have been unable to reproduce these results by using similar techniques. In addition, TWEAK-DR3 interaction could not be confirmed using slide binding assays performed either by overexpressing TWEAK in transfected cells and probing with DR3-IgG-Fc fusion protein, or by overexpressing DR3 in transfected cells and probing with TWEAK (data not shown). In agreement with these findings, recently published studies indicate that TWEAK does not bind DR3 and that a receptor specific for TWEAK must exist (Schneider et al., 1999; Kaptein et al., 2000).

In this paper we report the identification of a member of the TNF receptor family, TweakR, which binds to TWEAK with high affinity. Members of the TNF receptor family can be identified by a distinctive set of cysteine-rich repeat regions in the extracellular domain (reviewed in Locksley et al., 2001). Most receptors have three to four of these regions, although some, such as the receptors for TRAIL/Apo2L and TACI have only two. One known member of the TNF receptor family, BCMA, has been described that has only one such region (Madry et al., 1998). TweakR also has just one cysteine-rich region in the extracellular domain, and with a total amino acid count of 102 (after signal peptide cleavage), it is the smallest TNF receptor family member so far described.

While this manuscript was in preparation, Feng et al. (2000) reported the cloning, chromosomal location, and expression properties of a human gene named Fn14. The Fn14 and TweakR nucleotide sequences have 100% identity. The Fn14 gene was first identified using a differential display approach to search for growth factor-inducible genes in murine NIH 3T3 fibroblasts, and Fn14 itself was shown to be a plasma membrane-anchored protein (Meighan-Mantha et al., 1999). However, due to the degree of amino acid sequence divergence from other TNF receptor family members, Fn14 was not classified as a member of the TNF receptor family. Here we demonstrate that this relatively small molecule is a fully functional member of the TNF receptor family able to transduce a signal to its host cell, and implicate TweakR in a variety of vascular cellular responses both *in vitro* and *in vivo*.

Results

Expression Cloning of TweakR from HUVECs

An expression cloning panning approach was used to identify a TWEAK binding receptor expressed in HUVEC. Magnetic beads were coated with the C-terminal receptor binding domain of TWEAK and used for two rounds of panning of COS cells transfected with a HUVEC cDNA expression library. The resulting enriched pool was further broken down by slide binding (Goodwin et al., 1993), in order to identify a single clone encoding the TWEAK binding activity. Sequence analysis of the recovered clone predicted TweakR to be a type-I transmembrane protein with a single extracellular cysteine-rich region comprising six cysteine residues in its extracellular do-

⁴ Correspondence: wileys@immunex.com

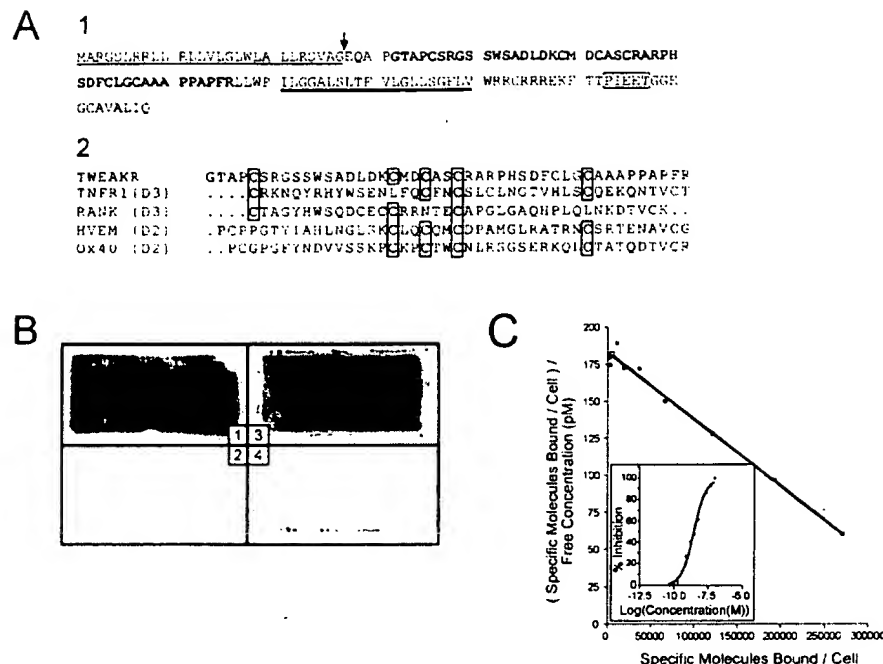


Figure 1. Features and Homology of Predicted TweakR Primary Amino Acid Sequence

(A1) Predicted primary amino acid sequence of TweakR showing major features. The leader sequence is underlined. The arrow indicates the predicted site of cleavage of the leader sequence. The region of TNF family receptor homology is shown in bold. The predicted transmembrane region is doubly underlined. The putative TRAF binding motif in the cytoplasmic domain is boxed.

(A2) Alignment of TweakR to selected cysteine-rich domains of other members of the TNF receptor family. The family member and cysteine-rich domain number are indicated on the left. Boxes emphasize aligned cysteines.

(B) Slide binding showing qualitative interaction of TweakR with TWEAK. Shown is an autoradiographic image of Cos cells transfected with TweakR versus control vector and probed with TWEAK-LZ and 125 I-labeled M15 antileucine zipper antibody (1 and 2, respectively), or CV1-EBNA cells transfected with human full-length TWEAK versus control vector and detected with TweakR-Fc and 125 I-labeled goat antimouse IgG antibody (3 and 4, respectively).

(C) CV-1 cells transfected with human full-length TWEAK were mixed at a 1:30 ratio with Raji cells and incubated with various concentrations of 125 I-labeled TweakR-Fc as described in the Experimental Procedures. Shown is a Scatchard representation of specific binding. (Inset) Plot of competitive inhibition of unlabeled versus 125 I-labeled TweakR-Fc.

main (Figure 1, A1). An alignment of the cysteine-rich extracellular region with the cysteine-rich region of other TNF receptor family members revealed a weakly conserved pattern of extracellular cysteines (Figure 1, A2). The predicted twenty-eight amino acid cytoplasmic domain contains a region, which bears resemblance to known binding sites for TRAF family signaling molecules (see below). Aside from the cysteine pattern, and the putative TRAF binding site, there is virtually no sequence conservation between TweakR and other TNF receptor family members. TWEAK-TweakR interaction was qualitatively confirmed by slide-binding assays conducted by either transfecting the isolated TweakR cDNA clone into cells and detecting with TWEAK-LZ and 125 I-labeled antileucine zipper antibody, or conversely by transfecting a full length TWEAK cDNA clone into cells and detecting by TweakR-Fc and 125 I-goat antihuman IgG antibody (Figure 1B).

TWEAK Ligand-Receptor Affinity

Given the unusually small size of TweakR, and the lack of strong similarity to other TNF family receptors, it was necessary to determine the affinity of its interaction with

TWEAK. The intrinsic affinity between TWEAK ligand and receptor was estimated by measuring the equilibrium binding constant between TweakR-Fc and recombinant full-length surface ligand transiently expressed on CV1/EBNA cells. Direct binding of 125 I-TweakR-Fc to surface ligand, plotted in Scatchard format (Figure 1C), gave an affinity constant of 2.3 ± 0.1 nM. Consistent with this value, competitive inhibition assays with unlabeled TweakR-Fc gave a $K(i)$ of 2.5 ± 0.1 nM (Figure 1C, inset). Confirmation that TweakR is largely responsible for TWEAK binding to native cells was demonstrated by the ability of a monoclonal antibody against TweakR to significantly inhibit binding of TWEAK to endothelial cells as measured by flow cytometry (data not shown). Together these experiments represent five independent experimental approaches demonstrating a TWEAK-TweakR interaction.

The TweakR Cytoplasmic Domain Binds TRAF1, 2, and 3

Many TNF receptor family members have been shown to bind members of the TRAF family of signal transduction molecules. This, combined with the observation that

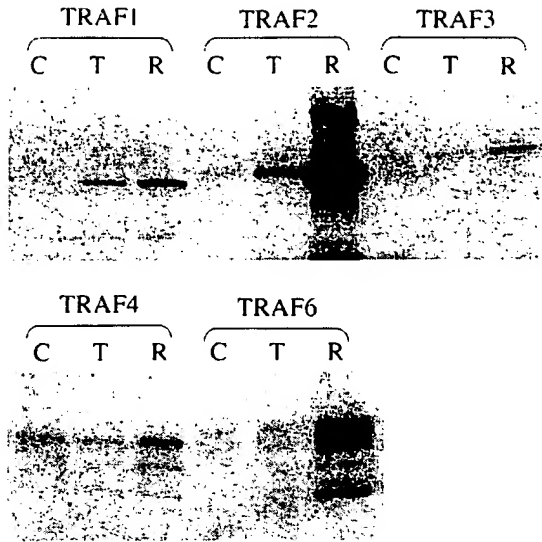


Figure 2. Qualitative Binding of TRAFs to the TweakR Cytoplasmic Domain

Indicated TRAF molecules were 35 S-labeled by coupled *in vitro* transcription/translation and incubated with GST beads (C), GST-TweakR cytoplasmic domain fusion beads (T), or GST-RANK cytoplasmic domain fusion beads (R). Beads were washed and the bound TRAF molecules were resolved by SDS-PAGE and visualized by autoradiography.

the TweakR cytoplasmic domain contains a region that resembles TRAF binding motifs found in many TNF family receptors, led us to qualitatively test the ability of this region to bind to various members of the TRAF family. The 28 amino acid TWEAK cytoplasmic domain was fused to glutathione S-transferase (GST) and then bound to glutathione-Sepharose beads. Control beads bound with GST lacking the TweakR cytoplasmic domain were used to determine background levels of binding. Beads bound with the cytoplasmic domain of the TNF receptor family member RANK fused to GST were used as a positive control. This RANK-GST fusion construct is a high affinity TRAF binder that contains two sites for TRAF interaction and binds to TRAF1, 2, 3, 5, and 6 (Galibert et al., 1998). The beads were incubated with *in vitro* translated 35 S-labeled TRAF proteins. The results show that under the conditions used TRAF1 and TRAF2 can interact with the TWEAK cytoplasmic region GST coated beads above background levels (Figure 2). TRAF3 binding is weaker, but still above background levels. TRAF4 and TRAF6 showed no binding to the TweakR cytoplasmic domain above background levels. TRAF5 did not express well in our *in vitro* system, and therefore the binding of this protein to the TweakR cytoplasmic tail could not be evaluated.

Cross-Linking of TweakR Transmits a Proliferative Signal to HUVEC

Although TweakR binds to TWEAK with an affinity that is consistent with other TNF ligand-receptor interactions, these data do not demonstrate that TweakR functions as a receptor, which can transduce a signal into the host

cell. For example, it is formally possible that TweakR actually inhibits rather than promotes TWEAK signaling. In order to investigate whether or not the molecule resulting from the panning experiment was a functional receptor, a construct was made that fuses a synthetic Flag octapeptide epitope onto the N-terminal extracellular domain of TweakR (Flag-TweakR). The resulting protein was expressed by transient transfection in HUVEC and incubated with cross-linked antiFlag monoclonal antibody. Cross-linking the receptor in this manner avoids background from the endogenous TweakR expressed by HUVEC. Proliferation was measured by BrdU incorporation into DNA. Lipid mediated transfection of HUVEC with Flag-TweakR resulted in expression of recombinant Flag-TweakR on the cell surface by 36 hr posttransfection. The Flag-TweakR was expressed at a high level by 9% of the transfected HUVEC used in these experiments, with a mean fluorescence intensity (MFI) of 99 compared to the MFI of the vector only HUVECs of 4.5. The results are expressed as the fold increase in the number of BrdU positive cells (BrdU mAb-FITC positive cells) under each culture condition over the number of negative-control FITC positive cells. *In vitro* culture of Flag-TweakR expressing HUVEC with the complex of M2 antiFlag and goat antimouse IgG increased BrdU incorporation 3-fold over the level of BrdU incorporation observed by culturing Flag-TweakR expressing cells with goat antimouse IgG alone (Figure 3). Culture of Flag-TweakR expressing HUVEC with the complex of M2 antiFlag and goat antimouse IgG increased BrdU incorporation 6-fold over the level of BrdU incorporation observed by culturing vector alone-transfected HUVEC with the cross-linking complex. Incubation with the cross-linking complex did not alter BrdU incorporation in vector alone-transfected HUVEC. The 2-fold increase in BrdU incorporation between cells expressing Flag-TweakR relative to control cells in the absence of cross-linking is likely because of a low level of spontaneous signaling due to overexpression of the receptor. These data provide direct evidence that despite its small size, TweakR is capable of initiating a proliferative signal in human endothelial cells.

Regulation of TweakR mRNA Expression in Vascular SMC

Previous studies have demonstrated that growth factor stimulation of quiescent murine or human fibroblasts promotes a transient increase in TweakR (Fn14) gene expression (Meighan-Mantha et al., 1999; Feng et al., 2000). In consideration of the ability of TWEAK to stimulate proliferation of endothelial and smooth muscle cells (Lynch et al., 1999), we investigated whether TweakR gene activation also occurred in mitogen-stimulated vascular cells. First, rat aortic SMC were serum-starved and then treated with FGF-2 for various lengths of time. RNA was isolated and TweakR mRNA levels were examined by Northern blot hybridization. A single TweakR transcript of ≈ 1.2 kb in size was detected in SMC. TweakR mRNA expression was transiently induced following FGF-2 addition, with maximal levels detected at 2 hr post-stimulation (Figure 4A). Second, we treated serum-starved SMC for 4 hr with various agents (e.g., phorbol ester, polypeptide growth factors, peptide

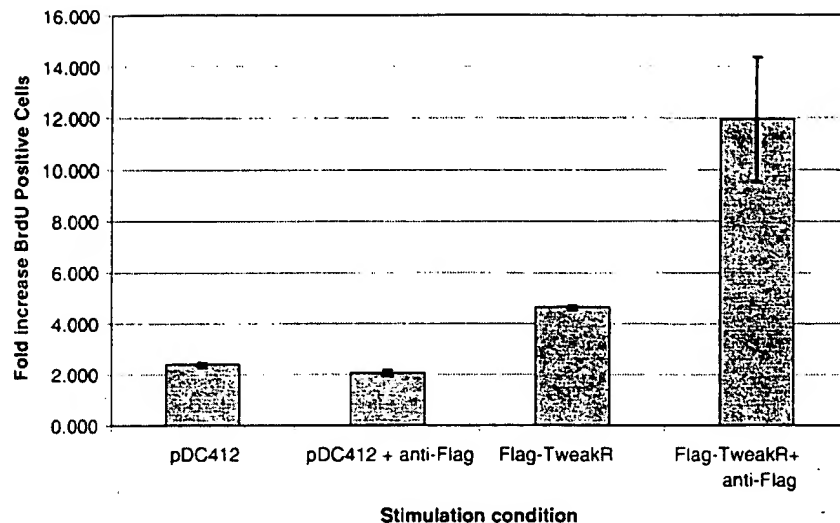


Figure 3. BrdU Incorporation into HUVECs Transfected with Either Empty Vector (pDC412) or a Flag Epitope-Tagged TweakR
Triplicate cultures of cells transfected as indicated were incubated in media containing goat-antimouse IgG in either the presence or absence of M2 antiFlag mAb. The results are expressed as the mean fold increase \pm SEM in BrdU positive cells compared to control for each culture condition.

hormones), and then performed Northern blot analysis to determine whether TweakR gene expression could be induced by multiple, distinct, growth promoters. TweakR mRNA levels were significantly elevated above unstimulated levels following PMA, FBS, PDGF-BB,

EGF, FGF-2, or Ang II treatment of rat SMC (Figure 4B). In comparison, TGF- β 1, IGF-1, or α -thrombin treatment had only a slight stimulatory effect. These results indicate that TweakR is a growth factor-regulated gene in vascular SMC.

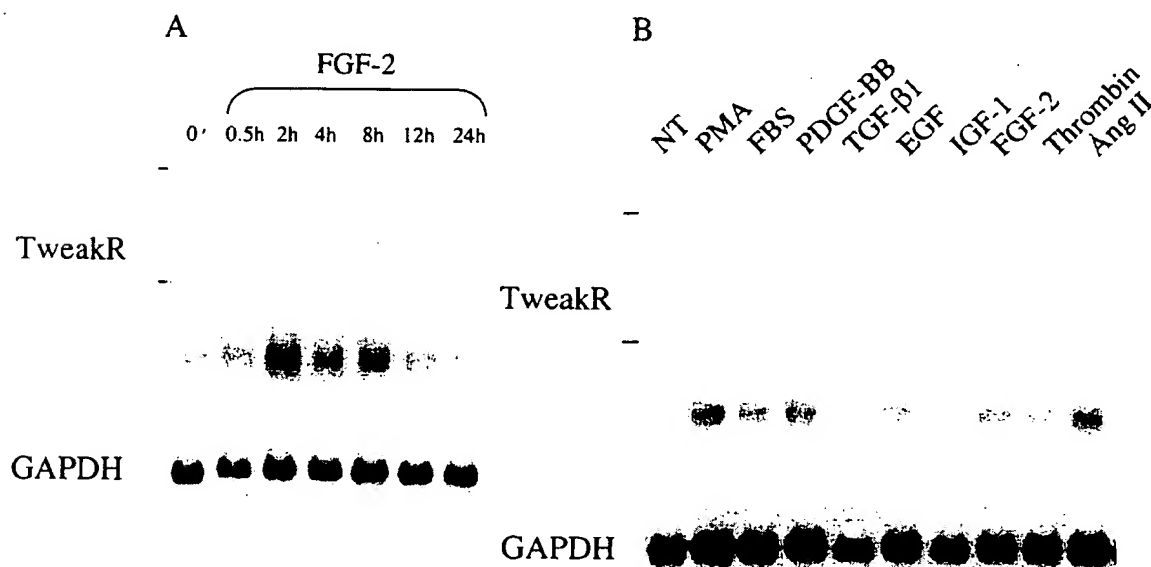


Figure 4. Regulation of TweakR mRNA Expression in Rat Aortic SMC

(A) Serum starved cells were either left untreated (0') or treated with FGF-2 for the indicated time periods. RNA was isolated, and equivalent amounts of each sample were analyzed by Northern blot hybridization using the two cDNA probes indicated. The positions of 28S and 18S rRNA are noted on the left with tick marks.

(B) Serum starved cells were either left untreated (NT, no treatment) or treated with phorbol myristate acetate (PMA), fetal bovine serum (FBS), PDGF-BB, TGF- β 1, EGF, IGF-1, FGF-2, α -thrombin, or angiotensin II (Ang II) for 4 hr. RNA was isolated, and equivalent amounts of each sample analyzed by Northern blot hybridization.

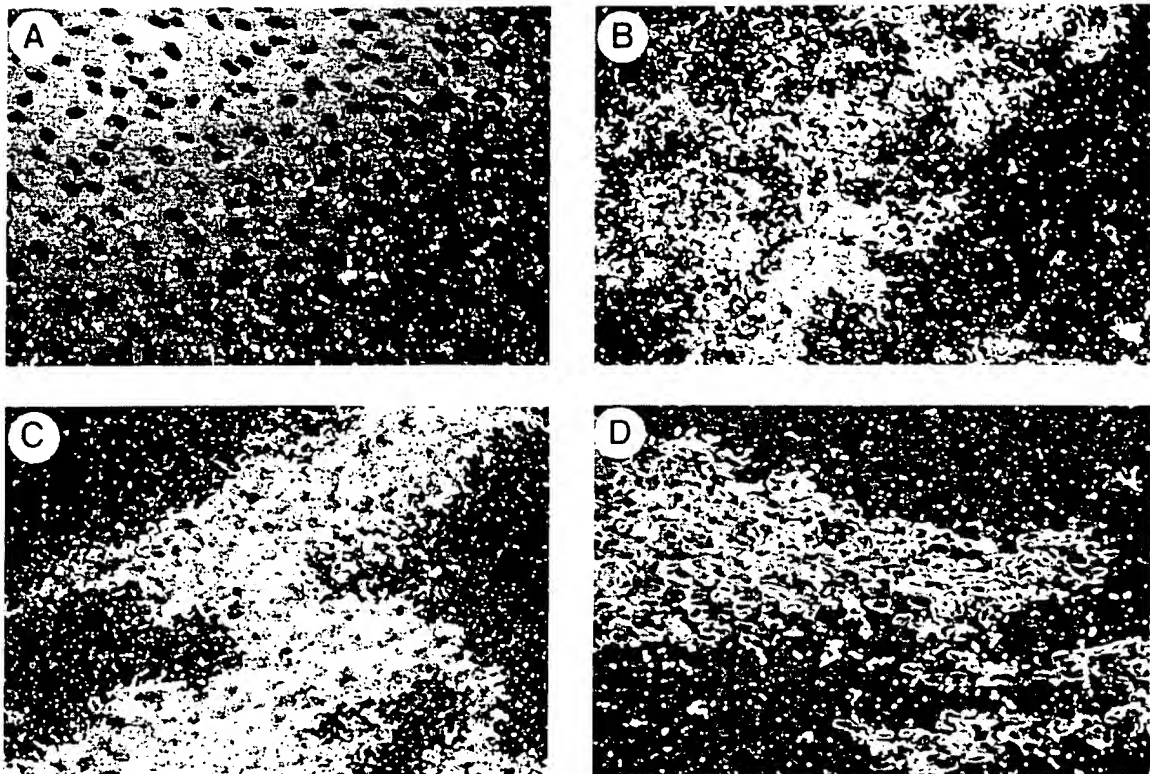


Figure 5. Photomicrographs of En Face Preparations of Rat Aortae Examined for TweakR Expression by In Situ Hybridization with 35 S-UTP Labeled Antisense and Sense Probes

- (A) The uninjured aorta showed low levels of TweakR expression in the endothelium.
 (B) Proliferating and migrating endothelium at the wound edge 8 days after aortic injury revealed upregulated expression of TweakR. The still denuded area is located on the right.
 (C) Strong expression of TweakR mRNA was seen in proliferating SMC forming the neointima at 8 days following balloon injury.
 (D) Hybridization with the sense probe showed very little background hybridization in SMC 8 days after injury. Hematoxylin was used for staining nuclei, and all specimens were viewed under dark field illumination at 200 \times original magnification.

TweakR mRNA Expression in Injured Rat Arteries

A common clinical situation that involves proliferation of both smooth muscle and endothelial cells results from vessel damage induced by balloon angioplasty. Therefore, we examined TweakR mRNA expression following balloon catheter denudation of rat carotid arteries. In situ hybridization of en face preparations using 35 S-labeled riboprobes allowed us to compare TweakR mRNA expression levels in quiescent vs. proliferating EC, and also to examine TweakR mRNA expression in SMC accumulating on the luminal surface after injury. We found that endothelium from uninjured arteries expressed low levels of TweakR mRNA (Figure 5A); however, significantly higher levels of expression were detected in proliferating EC at the wound edge (Figure 5B). In addition, high levels of TweakR mRNA expression were found in proliferating, intimal SMC at 8 days after injury (Figure 5C). All en face preparations probed with a 35 S-labeled TweakR sense riboprobe as a control revealed low levels of hybridization (Figure 5D). These results indicate that TweakR mRNA expression is upregulated in proliferating EC and SMC in vivo.

TweakR-Fc Inhibits Migration of HRMECs In Vitro

Angiogenesis is a multi-step process that requires not only proliferation, but also morphological alterations and migration of vascular cells. Therefore, the effect of blocking TweakR signaling on endothelial cell migration was tested by use of an in vitro planar endothelial cell migration (wound closure) assay (Daniel et al., 1999). In this assay, migration of primary human renal microvascular endothelial cells (HRMEC) is measured by the rate of closure of a circular wound in a cultured cell monolayer. The rate of wound closure is linear, and is dynamically regulated by agents that stimulate and inhibit angiogenesis in vivo. By fusing the extracellular portion of TweakR to the human IgG1-Fc domain (TweakR-Fc), a soluble inhibitor of TweakR signaling was created. Two stimuli were used to increase the rate of closure of the HRMEC: PMA or EGF (Figure 6A and 6B). TweakR-Fc inhibited PMA-stimulated endothelial cell migration in a dose responsive manner, reducing the rate of migration to near basal levels at 1.5 μ g/ml. Neither hulgG-Fc nor TweakR-Fc added alone inhibited basal (uninduced) migration (Figure 6A). EGF-induced HRMEC migration was

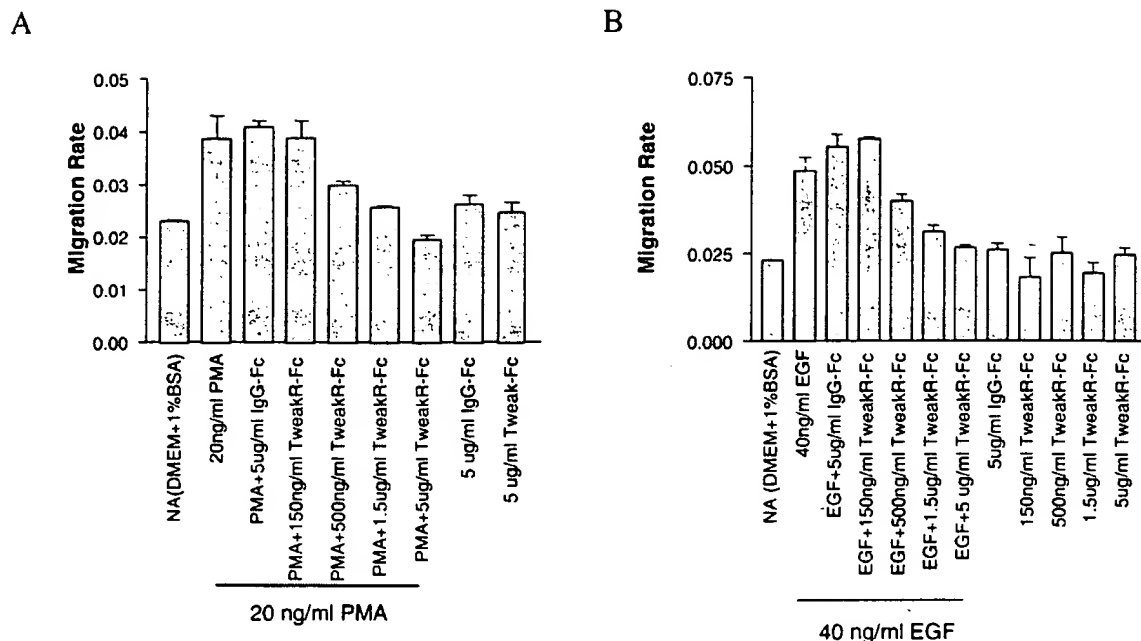


Figure 6. Human TweakR-Fc Inhibits PMA- or EGF-Stimulated Endothelial Cell Migration In Vitro

(A) At the time of wound initiation, basal media was supplemented with PMA, PMA + huIgG-Fc, PMA + titrated TweakR-Fc, huIgG-Fc alone, or huTweakR-Fc alone at the concentrations indicated. After 12 hr of incubation the means of the residual wound areas, expressed as fractions of the original wound, were converted into migration rate (expressed as the percentage of the original wound area covered per hour). The results expressed in the columns are the mean migration rate for each condition (triplicate cultures) at 12 hr ± SEM (error bars). (B) At the time of wound initiation, basal media was supplemented with EGF, EGF + huIgG-Fc, EGF + titrated TweakR-Fc, huIgG-Fc alone, or TweakR-Fc alone at the concentrations indicated. The experiment was performed and analyzed as described in (A).

similarly inhibited by TweakR-Fc, reducing the rate of migration to unstimulated levels at 5 μ g/ml (Figure 6B). These results indicate that endogenous TWEAK regulates endothelial cell wound closure rate in this assay.

TweakR-Fc Inhibits FGF-2-Stimulated Corneal Angiogenesis In Vivo

In order to test the relevance of signaling by TweakR in vivo, TweakR-Fc was tested for its ability to inhibit FGF-2-induced angiogenesis in a mouse cornea pocket assay (Figure 7). In this assay, agents to be tested for angiogenic or antiangiogenic activity are immobilized in a slow release form in a hydron pellet, which is implanted into micropockets created in the corneal epithelium of anesthetized mice. Quantitation is measured by photography using a slit-lamp microscope followed by analysis with image processing software. Vascularization is measured as the area, density, and extent of vessel growth from the vascularized corneal limbus into the normally avascular cornea. The results show that local administration of TweakR-Fc (100 pmol) inhibited FGF-2-stimulated corneal angiogenesis (Figure 7A), reducing the vascular area to 50% of that induced by FGF-2 alone or FGF-2 plus control-IgG-Fc (Figure 7B). In addition to reducing vascular area, local administration of TweakR-Fc significantly inhibited FGF-2-stimulated induced vessel density (imaged on hemoglobin) by 70% compared to the vessel density in the presence of the control pro-

tein IgG-Fc. This demonstrates that TweakR signaling plays a role in certain types of angiogenesis in vivo.

Discussion

The results presented here describe the identification and initial functional characterization of a receptor for TWEAK in endothelial cells. The TweakR is a type-I transmembrane protein of 128 amino acids in length, making it the smallest of all known TNF receptor family members. It has only one cysteine-rich domain in the extracellular region, a feature shared with just one other known TNF receptor, BCMA (Madry et al., 1998). The short 28 amino acid putative cytoplasmic domain contains a sequence that is similar to TRAF binding motifs found in other TNF family receptors. However, these features are only clear in hindsight. Based on the primary amino acid sequence alone, it is very difficult to identify TweakR as a member of the TNF receptor family, or even as a receptor of any kind. An alignment of the cysteine-rich region of TweakR with those of other TNF receptor family members demonstrates the high degree of divergence from other family members (Figure 1, A2).

Despite this unusually small extracellular domain, and divergence in the cysteine-rich repeat region, the K_a of TWEAK-TweakR interactions ($4.2 \times 10^8 \text{ M}^{-1}$) is similar to the affinity of CD30 to its receptor (4.0×10^8) when measured the same way (Smith et al., 1993). Although

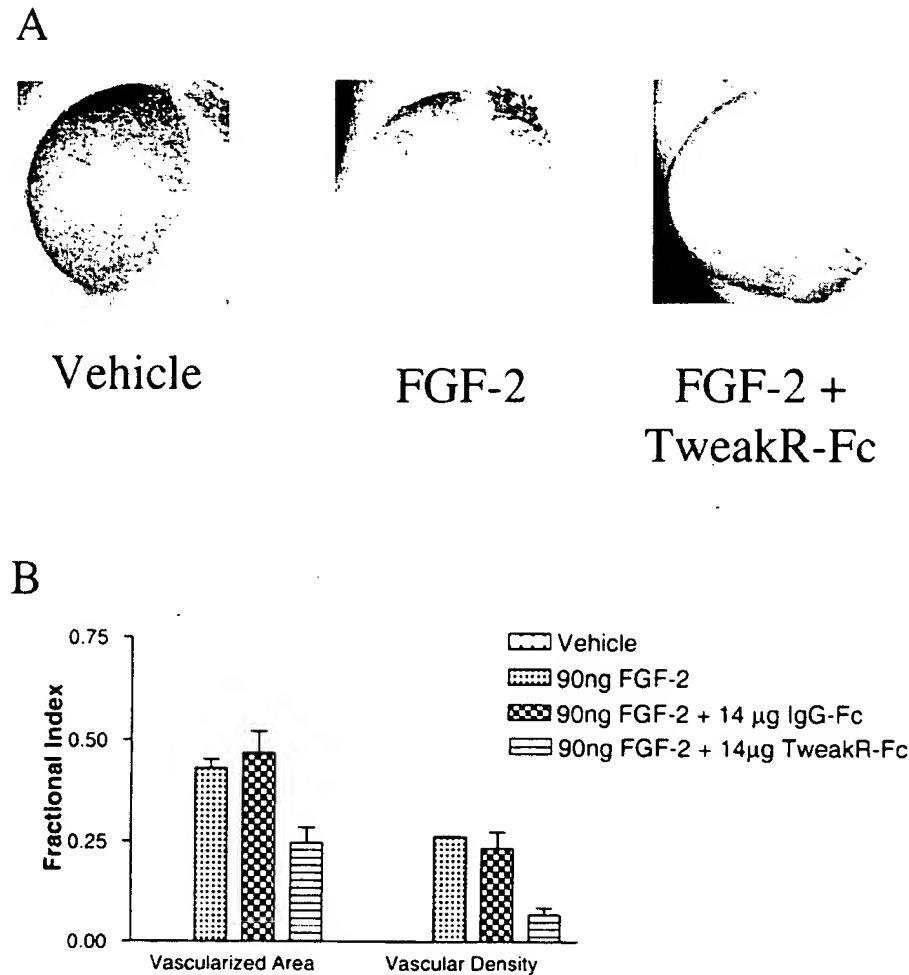


Figure 7. TweakR-Fc Treatment Reduces FGF-2-Stimulated Corneal Angiogenesis in the Mouse

(A) Representative photographs illustrating corneal vessel growth in vehicle only containing pellets as compared to localized administration of FGF-2 and concomitant administration of FGF-2 and TweakR-Fc.

(B) The results were quantified as described in Experimental Procedures. The fractional vascularized area of the cornea and the total vascular density within the corneal perimeter induced by FGF-2 in the presence of no inhibitor, hulgG or TweakR-Fc is shown (columns = mean, error bars = SEM). A significant decrease in new corneal vessels is observed with concomitant administration of FGF-2 and TweakR-Fc ($p = 0.0028$). Values for each experimental condition are obtained from a total of nine separate animals.

this affinity is approximately 20-fold less than that measured for TNF binding to TNF-R1, it is still in the physiological range for this family of ligand-receptor cognates. Also, the binding curve produced by the analyses is a monovalent curve, as opposed to the bivalent curve sometimes seen in similar analyses with other TNF family members. This indicates that only one type of binding site was detected, rather than a mixture of high and low affinity sites.

The 28 amino acid cytoplasmic domain of TweakR is also much smaller than many other receptors in the family. Nevertheless, the cytoplasmic region is capable of binding TRAF family members 1, 2, and 3 (Figure 2). Although this does not rule out other potential signaling pathways, one possible mechanism for TweakR signaling is through the TRAF family of adaptor proteins. After transfection into endothelial cells, full-length TweakR

transduces a proliferative signal into the cell following receptor cross-linking (Figure 3). Cross-linking via an extracellular artificial epitope, rather than using the natural ligand, ensures that the increase in proliferation is due to signaling by TweakR, as opposed to some other hypothetical receptor for TWEAK. Therefore, despite its small cytoplasmic region, TweakR is a functioning receptor.

Northern blot analyses show that TweakR gene expression is rapidly upregulated in smooth muscle cells by a variety of agents and known growth factors (Figure 4). This leaves open the possibility that TweakR might be involved in a range of biological activities. One such activity is endothelial cell migration, which is a necessary component of angiogenesis. The rate of wound closure in a HRMEC monolayer is enhanced by multiple stimuli such as PMA and EGF. Blocking TweakR reduces

this enhanced rate of wound closure back to unstimulated levels (Figure 6). Presumably, this is due to inhibition of a TWEAK-TweakR autocrine loop. This possibility is supported by RT-PCR data indicating that these cells coexpress TWEAK and TweakR transcripts (data not shown). Failure of TweakR-Fc to inhibit the basal rate of closure suggests that induction of the TweakR pathway is a required component of this autocrine loop. The ability to neutralize this enhanced rate of closure is evidence that TweakR is a required part of the activation seen by PMA and EGF. Interestingly, the basal rate of wound closure is not significantly affected by TweakR-Fc, demonstrating that the basal response is not dependent on TweakR signaling.

We also found that FGF-2 angiogenic activity can be partially blocked by inhibition of TweakR signaling *in vivo* (Figure 7). Again, this implies that at least in part, a TWEAK-TweakR interaction mediates some of the FGF-2 effect. In particular, the effect of blocking TWEAK-TweakR signaling was more dramatic on vessel density than it was on overall vessel area.

TweakR mRNA expression is induced during tissue regeneration, both during repair of a denuded rat artery (Figure 5), and during liver regeneration (Feng et al., 2000). A pattern that seems to be emerging is that TweakR expression is upregulated in growing or regenerating tissues. Whether this expression is largely restricted to endothelial and smooth muscle cells is not clear. TweakR, for instance, is also present in fibroblast cells (Meighan-Mantha et al., 1999; Feng et al., 2000), and TWEAK can induce a signal in astrocytes (Saas et al., 2000). However, additional *in situ* hybridization data showing the cellular localization of TweakR expression in developing and regenerating tissues must be generated in order to help clarify the role of this molecule. Regardless of its presence in cell types not directly associated with vasculature, data presented here argue for a role for TweakR in vascular cell migration and angiogenesis.

Two other members of the TNF family have also previously been implicated in angiogenesis: TNF and Fas ligand (Pandey et al., 1995; Biancone et al., 1997). However, these effects are thought to be indirectly mediated by production of pro-angiogenic endothelial growth factors (Yoshizumi et al., 1992). In contrast, the proliferative effect of TWEAK on endothelial cell growth is not correlated with TWEAK-induced expression of known pro-angiogenic factors or their receptors (Lynch et al., 1999). This implies that the angiogenic effect of TWEAK in a cornea pocket angiogenesis assay is a direct effect rather than one mediated by better characterized angiogenic factors such as VEGF and FGF. However, the effects of some other angiogenic factors may be mediated in part by TWEAK-TweakR interactions. For example, both FGF-2 and EGF can upregulate TweakR expression in fibroblasts (Meighan-Mantha et al., 1999) and smooth muscle cells (Figure 4). We have shown that blocking TweakR signaling inhibits both EGF-stimulated endothelial cell wound closure (Figure 6B) and FGF-2-induced cornea angiogenesis (Figure 7).

In summary, TweakR, despite its small size, is a fully functional receptor for TWEAK, capable of both binding TWEAK at a reasonable affinity and of transducing a proliferative signal to endothelial cells. A variety of data,

both *in vitro* and *in vivo*, supports the hypothesis that the TWEAK-TweakR system plays a role in endothelial cell growth and migration. This system may be important for both the biological activity of the TWEAK ligand itself, as well as for the biological activity observed with other more thoroughly characterized proangiogenic molecules.

Experimental Procedures

Cell Culture

Adult rat (Sprague-Dawley) thoracic aorta SMC were kindly provided by M. Majesky, Baylor College of Medicine, Houston, Texas. The cells were cultured at 37°C in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium (Mediatech) supplemented with 5% fetal bovine serum (FBS; Hyclone Laboratories), 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B (JRH Biosciences). SMC cultures were fed every 48 hr and expanded by trypsin-EDTA (JRH Biosciences) treatment and subculturing at a 1:5 split ratio. Cells were incubated for ~72 hr in cell culture medium containing 0.5% FBS to obtain a relatively quiescent SMC population. Serum-starved cells were then either left untreated or treated with one of the following: 10 ng/ml human recombinant FGF-2 (Bachem), 10 ng/ml human recombinant PDGF-BB (Genzyme), 2 ng/ml human recombinant TGF-β1 (R&D Systems), 10 ng/ml human recombinant IGF-1 (Bachem), 10% FBS, 20 ng/ml phorbol myristate acetate (PMA; Sigma), 10 ng/ml EGF (Genzyme), 10⁻⁸ M α-thrombin (Sigma), or 10⁻⁸ M angiotensin II (Ang II; Bachem). Primary human renal microvascular endothelial cells, HRMEC, were isolated, cultured, and used at the third passage after thawing as described (Martin et al., 1997). All other cell lines were cultured to a density of 2–5 × 10⁵ cells per ml in RPMI medium supplemented with 10% fetal bovine serum, 100 µg/ml streptomycin, and 100 µg/ml penicillin.

Plasmid Construction and Expression

pDC412-LZ-TWEAK, the soluble form of human TWEAK, was constructed with a modified leucine zipper (LZ) on the N terminus preceded by the growth hormone leader. The construct was made essentially like the previously described soluble LZ-CD40 ligand (Fanslow et al., 1994) in the mammalian expression vector pDC412, a derivative of pDC409 (Wiley et al., 1995) that reverses the order of the BglII and NotI sites in the multiple cloning vector. Flag-TweakR was created by fusing the leader sequence of the murine Ig α-chain (amino acids 1–21) to a Flag synthetic epitope, and abutting to the N-terminal end of the mature TweakR (amino acids 35–129), which was then placed in the pDC412 vector. TweakR-Fc was produced by placing amino acids (1–79) of TweakR into the SalI-BglII site of the pDC412-Fc vector (Smith et al., 1993). Expression and purification of the TweakR-Fc protein was performed essentially as described (Goodwin et al., 1993).

Expression Cloning of TweakR cDNA

pDC409-LZ-TWEAK conditioned supernatants were produced by transient transfection into CV1-EBNA cells. These supernatants were incubated with magnetic beads coated with polyclonal goat antimouse antibody (Ambion) that had previously been incubated with a mouse monoclonal antibody against the leucine zipper (M15, 5 µg/ml). Control beads were produced by mixing the M15 coated beads with supernatants from cells transfected with empty vector. A monolayer of COS cells, grown in a T175 flask, was transfected with 15 µg of DNA from a HUVEC cDNA expression library (Edge Biosystems). The complexity of the DNA pools was ~100,000. After 2 days these cells were lifted from the flask by nonenzymatic means (Cell Dissociation Solution; Sigma) and incubated in 1.5 ml of binding media (Goodwin et al., 1993) plus 5% nonfat dried milk for 3 hr at 4°C on a rotator wheel. Cells were precleared by adding 1 µg of control beads and rotated at 4°C for an additional 45 min, after which bead-bound cells were removed with a magnet. Preclearing was repeated 2 to 3 times before 1 µg of the TWEAK-coated beads was added to the cells, which were then rotated 30 min at 4°C. Cells binding the TWEAK beads were separated by use of a magnet and

washed four times in PBS. Plasmid DNA was extracted from these cells by lysing in 0.1% SDS and used to transform the *Escherichia coli* strain DH101B. Colonies were grown 16 hr on ampicillin selective media. Transformants were pooled and used as a source of plasmid DNA pools for the next round of panning. After two rounds of panning, the positive clones were picked from the resulting pool using a slide binding protocol. Slide binding was performed as described (Goodwin et al., 1993), with the exception that TweakR positive slides were detected by incubation with pDC412-LZ-Tweak conditioned supernatants followed by incubation with 125 I-labeled M15 antileucine zipper.

TRAF Binding

TRAF binding to cytoplasmic domain GST fusion proteins was performed as described (Galibert et al., 1998) except that the 35 S-labeled TRAFs were produced using a coupled in vitro transcription and translation system (TNT Labeling Kit, Promega Corp., Madison, Wisconsin).

Receptor-Ligand Binding Assays

Equilibrium binding isotherms between 125 I-TweakR-Fc and surface TWEAK ligand were determined by standard methods (Smith et al., 1993). Briefly, CV1/EBNA cells transfected with full-length human TWEAK ligand were diluted 30-fold into Raji cells (TWEAK-negative), and the suspension (1.7×10^7 total cells/ml) incubated with serially diluted 125 I-TweakR-Fc (4.4×10^{15} cpm/mole) in a total volume of 150 μ l for 2 hr at 4°C. Duplicate aliquots of suspension were sampled, free and bound 125 I-TweakR-Fc determined, and the data plotted in Scatchard format. Competitive inhibition assays were performed similarly (Smith et al., 1996) with 0.1 nM 125 I-TweakR-Fc incubated with cells and increasing concentrations of unlabeled inhibitor (TweakR-Fc). Data were fitted to a single site competitive inhibition equation (Smith et al., 1993).

RNA Isolation and Northern Blot Hybridization

Total RNA was isolated from cultured SMC using RNA Stat-60 (Tel-Test) according to the manufacturer's instructions. Northern blot hybridization analysis was performed as described (Meighan-Mantha et al., 1999). The cDNA hybridization probes were mouse TweakR/Fn14, ≈ 1.0 kb EcoRI/XhoI fragment of pBluescript/mFn14, and human GAPDH, ≈ 0.8 kb PstI/XbaI fragment of pHcGAP (American Type Culture Collection).

Arterial Injury Model and In Situ Hybridization

Carotid arteries from anesthetized male Sprague-Dawley rats were denuded with a balloon catheter and perfusion fixed as described (Silverman et al., 1999). In situ hybridization was performed on en face preparations of vessel segments as described (Silverman et al., 1999). A pBluescript/Fn14 plasmid containing the murine Fn14 cDNA sequence without the 3' untranslated region and poly(A) tail was constructed using standard techniques. This plasmid was linearized with SmaI, transcribed with T7 polymerase to make the antisense probe or linearized with ApaI, blunt ended with T4 DNA polymerase 1, and then transcribed with T3 polymerase to make the sense probe. Vessel segments were treated with proteinase K (1 μ g/ml) for 15 min at 37°C, prehybridized for 2 hr at 55°C in 0.3 M NaCl, 20 mM Tris-HCl (pH 7.5), 5 mM EDTA, 1X Denhardt's solution, 10% dithiothreitol, and 50% formamide, and incubated with the appropriate [35 S]UTP-labeled riboprobe for 16 hr at 55°C. After washing, the slides were coated with autoradiographic emulsion (Kodak, NTB2), exposed for 2 weeks, and then developed. All specimens were examined under dark-field illumination after nuclear counterstaining with hematoxylin. Images were photographed and digitized.

BrdU Incorporation in Transfected HUVECs

Proliferating human umbilical vein endothelial cells (HUVEC) obtained from BioWhittaker-Clonetics (Walkersville, Maryland) were grown in EGM-2 media (Clonetics) and then subjected to lipid-mediated DNA transfection. HUVECs were plated overnight at 3×10^5 per well in six-well plates, in 2.0 ml of EGM-2 medium per culture. The following day, transfection solutions were prepared using polystyrene tubes. PerFect Lipid #7 (Invitrogen, Carlsbad, California) was added to serum free endothelial basal media (EBM; Clonetics)

at 30 μ g per 0.5 ml for each transfection and then incubated at room temperature for 30 min. The transfection DNA was added to separate tubes in serum free EBM at 5 μ g per 0.5 ml for each transfection. The lipid and DNA solutions were then combined to make 1.0 ml of solution per transfection, which produced a solution with a lipid-to-DNA ratio of six to one (30 μ g lipid to 5 μ g DNA). The solution was then incubated for 15 min at room temperature.

Cell culture media was aspirated from all wells containing HUVEC for transfection. Then 1.0 ml of the appropriate transfection solution was added to culture wells and an additional 1.5 ml of serum free EBM was added. The transfection then proceeded at 37°C for 6 hr at 5% CO₂. Upon completion of incubation, the transfection solution was aspirated, and two washes were performed using 2.0 ml of pyrogen free PBS per wash. EGM-2 media was added back at 2.0 ml per well, and cells were incubated for 36–40 hr. Small samples of transfected cells were taken to determine the level of Flag-TWEAK expression by flow cytometry using M2 antitag as a detection reagent.

The remaining HUVEC were used for proliferation assays in triplicate culture for each stimulation condition tested. First, anti-Flag M2 at 20 μ g/ml per well of culture media was cross-linked with a goat anti-mouse IgG (Southern Biotechnology Associates, Birmingham, Alabama) at 40 μ g/ml. This complex was preincubated for 15 min at room temperature and then added to culture (cross-linked M2). The culture medium was aspirated, the wells were washed once with pyrogen free PBS, and EBM plus 0.05% FBS was added back at 2.0 ml per well. The goat antimouse IgG alone or cross-linked M2 was then added to culture wells, and the assay was incubated for 3 days at 37°C and 5% CO₂. One day before harvest, BrdU (BrdU Flow Kit; BD Pharmingen, San Diego, California) at 10 μ M final concentration was added to the cultures according to the manufacturer's instructions. Cells were incubated an additional 24 hr before trypsinization for assessment of BrdU incorporation. BrdU incorporation was measured by flow cytometry using a FITC-conjugated antiBrdU antibody supplied with the kit. BrdU mAb staining was compared to control staining and binding results analyzed using a FACScan (Becton Dickinson). In our hands, transfection efficiencies for Flag-TweakR using Lipid 7 averaged 8%. Flow cytometric BrdU incorporation results were evaluated by intensity of antiBrdU mAb binding and the percentage of the cells that bound antiBrdU mAb.

Planar Endothelial Migration Assay

Replicate circular lesions or "wounds" of 600–800 micron diameter were generated in confluent HRMEC monolayers using a silicon tipped drill press. At the time of wounding, the medium (DMEM \square 1% BSA) was supplemented with 20 ng/ml PMA (phorbol 12-myristate 13-acetate), 4 ng/ml EGF, and 0.150–5 μ g/ml TweakR-Fc, or a combination of 40 ng/ml EGF and 0.150 to 5 μ g/ml TweakR-Fc. As a control for TweakR-Fc, some cells were treated with 5 μ g/ml IgG-Fc. The residual wound area was measured as a function of time (0–12 hr) using microscope and image analysis software (Bioquant, Nashville, Tennessee). The relative migration rate was calculated for each agent and combination of agents by linear regression of residual wound area plotted over time.

Corneal Pocket Assay

Hydron pellets (Kenyon et al., 1996) incorporated sucralfate with FGF-2 (90 ng/pellet), FGF-2 and IgG-Fc (14 μ g/pellet, control), or FGF-2 and TweakR-Fc (14 μ g). The pellets were surgically implanted into corneal stromal micropockets created by microdissection 1 mm medial to the lateral corneal limbus of 6- to 8-week-old male C57BL6 mice. At the peak of neovascular response to FGF-2 (5 d), the corneas were photographed, using a Zeiss slit lamp, at an incipient angle of 35–50° from the polar axis in the meridian containing the pellet. Images were digitized and processed by subtractive color filters (Adobe Photoshop 4.0) to delineate established microvessels by hemoglobin content. Image analysis software (Bioquant) was used to calculate the fraction of the corneal image that was vascularized, the vessel density within the vascularized area, and the vessel density within the total cornea as described (Daniel et al., 1999). Statistical analysis (non-paired T test) was performed using GraphPad Prism software 3.0 (GraphPad Software, Inc., San Diego, California).

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